Transcriptional regulation of the novobiocin biosynthetic gene cluster

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The aminocoumarin antibiotic novobiocin is a gyrase inhibitor formed by a *Streptomyces* strain. The biosynthetic gene cluster of novobiocin spans 23.4 kb and contains 20 coding sequences, among them the two regulatory genes *novE* and *novG*. We investigated the location of transcriptional promoters within this cluster by insertion of transcriptional terminator cassettes and RT-PCR analysis of the resulting mutants. The cluster was found to contain eight DNA regions with promoter activity. The regulatory protein NovG binds to a previously identified binding site within the promoter region located upstream of *novH*, but apparently not to any of the other seven promoters. Quantitative real-time PCR was used to compare the number of transcripts in a strain carrying an intact novobiocin cluster with strains carrying mutated clusters. Both in-frame deletion of the regulatory gene *novG* and insertion of a terminator cassette into the biosynthetic gene *novH* led to a strong reduction of the number of transcripts of the genes located between *novH* and *novW*. This suggested that these 16 biosynthetic genes form a single operon. Three internal promoters are located within this operon but appear to be of minor importance, if any, under our experimental conditions. Transcription of *novG* was found to depend on the presence of NovE, suggesting that the two regulatory genes, *novE* and *novG*, act in a cascade-like mechanism. The resistance gene *gyrB^R*, encoding an aminocoumarin-resistant gyrase B subunit, may initially be co-transcribed with the genes from *novH* to *novW*. However, when the gyrase inhibitor novobiocin accumulates in the cultures, *gyrB^R* is transcribed from its own promoter. Previous work has suggested that this promoter is controlled by the superhelical density of chromosomal DNA.

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INTRODUCTION

Genome mining, i.e. the sequencing of bacterial genomes in search of novel biosynthetic gene clusters, is a promising new strategy for the discovery of bioactive substances, most importantly antibiotics (Wilkinson & Micklefield, 2007; Zerikly & Challis, 2009). The principal bottleneck for the

success of this strategy is the step from sequence data, which can be readily obtained by highly automated procedures, to the production of the encoded secondary metabolites in sufficient quantities for pharmacological screening. This can be achieved, e.g. by heterologous expression of the gene clusters. For this purpose, an understanding of the regulation of the expression of bacterial secondary metabolic gene clusters is of great practical importance for future drug discovery.

The aminocoumarin antibiotic novobiocin is produced by *Streptomyces caeruleus* (syn. *Streptomyces spheroides*) and is a potent inhibitor of bacterial gyrase (Maxwell & Lawson, 2003). It has been licensed for the treatment of infections with Gram-positive pathogens in humans (Albamycin, Pfizer). The closely related aminocoumarins clorobiocin and coumermycin A₁, produced by different *Streptomyces* strains, are even more potent antibacterials (Maxwell &

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Abbreviations: EMSA, electrophoretic mobility shift assay; 4HPP, 4-hydroxyphenylpyruvate; qRT-PCR, quantitative RT-PCR.

A set of supplementary methods, describing electrophoretic mobility shift assays, a supplementary figure, showing quantitative real-time analysis of *Streptomyces coelicolor* strains, and two supplementary tables, listing bacterial strains, plasmids and cosmids used in this study, and primers used in this study, with references, are available with the online version of this paper.

Lawson, 2003). The biosynthetic gene clusters of all three antibiotics have been cloned and sequenced from the genuine producer strains, and their heterologous expression in *Streptomyces coelicolor* M512 has provided the basis for the generation of many new aminocoumarins by mutasynthesis and pathway engineering experiments (Heide *et al.*, 2008; Li & Heide, 2005).

The novobiocin biosynthetic gene cluster spans 23.4 kb and consists of 20 coding sequences. The functions of these genes have been elucidated (Fig. 1) (Li & Heide, 2004, 2006). *novHIJ* and *K* are responsible for the synthesis of the aminocoumarin moiety. *novQ* and *R* are responsible for the generation of the prenylated 4-hydroxybenzoyl moiety, and *novSTUV* and *W* for the generation of the deoxysugar. The gene products of *novL* and *novM* catalyse the linkage of these three moieties. *novN*, *novO* and *novP* encode enzymes which catalyse tailoring reactions, i.e. the carbamoylation and methylation of the novobiocin skeleton. *novF* is probably responsible for the supply of 4-hydroxyphenylpyruvate (4HPP), a precursor of both aromatic moieties of novobiocin. *gyrB^R* encodes a resistance gene.

The coding sequences for *novQ*, *novR* and *novS* overlap, suggesting a translational coupling of these genes (Fig. 2k). The same is true for *novV* and *novW*, as well as for *novJ* and *novK*. The genes *novHIJ*, as well as *novKLM*, are separated by very short intergenic regions (≤ 19 bp). Large intergenic regions, suggestive of the presence of promoters, are found upstream of *novE* (180 bp intergenic region), *novG* (105 bp), *novH* (195 bp), *novO* (230 bp) and *gyrB^R* (376 bp) (Fig. 2k). The promoter activities of the DNA regions upstream of *gyrB^R* and upstream of *novE* have been shown by Thiara & Cundliffe (1989) and by Dangel *et al.* (2008), respectively. Eustáquio *et al.* (2005b) investigated the promoter situated upstream of *novH*, which contains the binding site for the positive regulator protein NovG. No NovG binding site is present in the large intergenic region upstream of *novO* (Eustáquio *et al.*, 2005b). In this study, we carried out additional electrophoretic mobility shift assays (EMSAs) with NovE (data not shown), but also failed to show binding to the region upstream of *novO*.

Notably, all 20 genes of the novobiocin biosynthetic gene cluster are arranged in the same orientation and many, or even all, of them could therefore be transcribed as a single operon. Bioinformatic sequence analysis shows no *rho*-independent terminators within the entire cluster.

The gene clusters of novobiocin, clorobiocin and coumermycin A₁ each contain a certain regulatory gene, termed *novG*, *cloG* or *couG*, respectively, which show sequence similarity to *strR*, a positive regulator of streptomycin biosynthesis (Tomono *et al.*, 2005). EMSAs have shown that NovG binds to a well-conserved inverted repeat located in the intergenic region between *novG* and *novH* (Eustáquio *et al.*, 2005b). This sequence is similar to the StrR binding sites in the streptomycin cluster, and to the binding sites of the closely related Bbr in the balhimycin cluster (Shawky *et al.*, 2007). These data suggest that NovG

and its orthologues CloG and CouG act as positive regulators of the transcription of the genes located downstream of their respective binding sites, although direct experimental proof of this hypothesis has not been provided so far.

Besides *novG*, the novobiocin gene cluster contains only one additional putative regulatory gene, i.e. *novE*. Orthologues of *novE* are found in the biosynthetic gene clusters of clorobiocin, coumermycin A₁, rubradirin and lincomycin (*cloE*, *couE*, *rubC4* and *lmbU*, respectively), but their function is as yet unknown. Inactivation of *novE* in a heterologously expressed novobiocin cluster leads to a strong reduction of novobiocin formation, and overexpression of *novE* leads to a twofold increase in production. Notably, inactivation of *novE* can be complemented by overexpression of *novG* (Dangel *et al.*, 2008). This suggests a role for *novE* as a positive regulator of novobiocin biosynthesis, although its influence on transcription of biosynthetic genes has not been demonstrated so far. No DNA binding activity of the NovE protein to the DNA region upstream of *novG* has been found in EMSAs (Dangel *et al.*, 2008).

In the present study, we investigated the transcriptional regulation of the novobiocin biosynthetic gene cluster. We present evidence that *novE* and *novG* act as transcriptional activators of novobiocin biosynthetic genes and that these two regulators act in a cascade-like mechanism.

METHODS

Bacterial strains, plasmids, cosmids and culture conditions.

Supplementary Table S1 shows the bacterial strains, plasmids and cosmids used in this study. The REDIRECT technology kit for PCR targeting (Gust *et al.*, 2003) was obtained from Plant Bioscience Limited.

Escherichia coli strains were cultivated in Luria–Bertani (LB) medium (Sambrook & Russell, 2001). *S. coelicolor* strains were routinely cultured in 300 ml baffled Erlenmeyer flasks containing a stainless steel spring and 50 ml yeast malt extract glucose (YMG) medium [1 % (w/v) malt extract, 0.4 % (w/v) yeast extract and 0.4 % (w/v) glucose (pH 7.3)]. Cultivation was carried out at 30 °C and 200 r.p.m. for 2 days. For preparation of protoplasts or isolation of genomic DNA, strains were grown in tryptone soya broth (TSB) medium (Kieser *et al.*, 2000).

For analysis of secondary metabolite production and RT-PCR experiments, 1 ml YMG preculture was inoculated into 300 ml baffled flasks containing 50 ml chemically defined minimal (CDM) production medium (Kominek, 1972), and cells were cultivated at 30 °C and 200 r.p.m.

For quantitative RT-PCR (qRT-PCR) experiments, strains were cultivated in 24-square deepwell plates (Duetz *et al.*, 2000). A 50 ml volume of YMG preculture, prepared as described above, was centrifuged (2772 g for 10 min). The cells were resuspended in 10 ml of 20 % (w/v) peptone (Bacto Proteose Peptone Number 3, Difco) and gently homogenized using a Potter homogenizer operated manually (B. Braun Biotech, Sartorius). The resulting mixture was divided into aliquots and stored at –70 °C. Frozen inoculum (2×10^6 c.f.u.) was mixed with 80 ml CDM production medium, containing 0.6 % (w/v) siloxylated polyether EO/PO copolymer Q2-5247 (Dow Corning), and 3 ml of this mixture was placed into each well of the

Fig. 1. Functions of the gene products of the 17 genes *novF* and *novHIJKLMNQRSTUUVW* in novobiocin biosynthesis.

Fig. 2. (a) RT-PCR detection of the transcripts of the novobiocin biosynthetic genes in the heterologous producer strain *S. coelicolor* M512(nov-BG1). (b–i) Insertion of the Ω_{aac} transcriptional terminator cassette into different genes of the novobiocin cluster and RT-PCR analysis of the resulting mutants. (k) Location of promoter regions in the novobiocin biosynthetic gene cluster. *gyrB^R*, novobiocin resistance gene. Numbers indicate the sizes of the intergenic regions (in bp).

24-square deepwell plates. Cultivation was carried out at 30 °C and 300 r.p.m.

Kanamycin (for *Streptomyces*, 15 µg ml⁻¹ in liquid medium and 50 µg ml⁻¹ in solid medium; for *E. coli*, 50 µg ml⁻¹), chloramphenicol (25–50 µg

ml⁻¹), apramycin (50 µg ml⁻¹), carbenicillin (50–100 µg ml⁻¹) and thiostrepton (8 µg ml⁻¹) were used for selection of recombinant strains.

In order to avoid methyl-sensing restriction, the recombinant plasmids and cosmids were amplified in *E. coli* ET12567 before

transformation into *S. coelicolor* strains (MacNeil *et al.*, 1992). The cultivation in production medium was carried out without addition of antibiotics.

DNA isolation, manipulation and cloning. Standard procedures for DNA isolation and manipulation were performed as described by Kieser *et al.* (2000) and Sambrook & Russell (2001). Genomic DNA was isolated from *S. coelicolor* strains by using the Kirby mix procedure (Kieser *et al.*, 2000). Southern blot analysis was performed on Hybond-N nylon membranes (Amersham Biosciences) with a digoxigenin-labelled probe by using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Molecular Biochemicals).

Plasmid construction. For the construction of pAE12, *novG* was amplified by PCR using pAE8 as template and the primer pair PG_for (5'-GAA GGT ACG GGA TCC CCA CG-3') and PG_rev (5'-GTC AGG CGG TGT CCC GGT C-3'). The PCR was carried out in a 50 µl volume with 100 ng template, 0.2 mM dNTPs, 50 pmol each primer and 5% (v/v) DMSO using an Expand High Fidelity PCR system (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 30 cycles with denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 90 s, and a final elongation step at 72 °C for 5 min. After purification, the PCR product was ligated into pGEM-T to give pAE11. The insert of pAE11 was checked by nucleotide sequencing. The construction of pJH1 and pAE12 is described in Supplementary Table S1. Plasmids were introduced into *S. coelicolor* M512(nov-BG1) by PEG-mediated protoplast transformation (Kieser *et al.*, 2000).

Insertion of the Ω aacC4 terminator cassette into cosmid nov-BG1 and heterologous expression of the modified cosmids. An apramycin-resistance cassette flanked by transcriptional terminators (Ω aacC4) was used for replacement of the second codon of the genes *novE*, *novF*, *novG*, *novH*, *novO*, *novP*, *novQ* or *novS* by λ Red-mediated recombination (Gust *et al.*, 2003). This resulted in cosmids nov-JH1 to nov-JH6, nov-JH10 and nov-JH12. The cassette for replacement was generated by PCR using the primers listed in Supplementary Table S2. The PCR was performed in a 50 µl volume with 50 ng template (pJH1 digested with *HinfI* and *SalI*), 0.2 mM dNTPs, 50 pmol each primer and 5% (v/v) DMSO, using the Expand High Fidelity PCR system (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 10 cycles with denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, extension at 72 °C for 90 s, and then 15 cycles with annealing at 55 °C, and a final elongation step at 72 °C for 5 min. *E. coli* XL1 Blue MRF' cells were analysed using restriction enzymes and gel electrophoresis. The generated cosmids were then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation (Kieser *et al.*, 2000). Kanamycin- and apramycin-resistant clones were checked for site-specific genomic integration of the cosmids into the Φ C31 attachment site by Southern blot analysis.

Analysis of secondary metabolites. Analysis of novobiocin production by HPLC was carried out as described previously (Eustáquio *et al.*, 2003).

RNA isolation and purification. For RT-PCR investigations, strains derived from *S. coelicolor* M512 were cultivated in 300 ml Erlenmeyer flasks as described above. After 72 h of cultivation the mycelia of 50 ml culture were collected by vacuum filtration.

For qRT-PCR experiments, strains were cultivated in 24-square deepwell plates (Duetz *et al.*, 2000) as described above. After 24, 32, 48, 72 and 96 h mycelia from four to six wells were pooled and collected by vacuum filtration. After 168 h, only three wells were harvested to analyse final novobiocin production.

Vacuum filtration was followed by rinsing of the cells with 30 ml distilled water. The cells were transferred to a 50 ml Falcon tube containing approximately 14 g 3.5–4.5 mm diameter glass beads and 15 ml modified Kirby mix [1% (w/v) *N*-lauroylsarcosine, 6% (w/v) sodium salicylate, 6% (v/v) phenol (buffered to pH 8) in 20 mM Tris/HCl (pH 8.3)] and vortexed for 2 min. The cell suspension was sonicated six times for 30 s with 20 s intervals (Branson Sonifier 250). To this mixture an equal volume (15 ml) of phenol:chloroform:isoamylalcohol (25:24:1) was added, followed by vortexing for 30 s and centrifugation (5000 g for 10 min at 4 °C). Clear supernatant was transferred to a clean 50 ml Falcon tube, mixed with an equal volume of 2-propanol and 0.1 volume of 3 M sodium acetate (pH 5.2), and left for 5 min at 20 °C. After centrifugation (5000 g for 20 min at 4 °C), the supernatant was discarded and the pellet was washed with 2 ml 70% (v/v) ethanol. The pellet was dried and resuspended in 800 µl water. After resuspension, a DNase treatment was carried out with DNase I (10 U; Fermentas) according to the manufacturer's instructions using 10 mM Tris/HCl (pH 7.5 at 25 °C), 2.5 mM MgCl₂, 0.1 mM CaCl₂ and RNase-free water. Subsequently, RNA was purified with the NucleoSpin RNA Clean-up kit (Macherey & Nagel) according to the manufacturer's instructions and eluted from the column with 40 µl water. RNA was quantified by determination of A₂₈₀. Integrity of RNA was checked by analysing 1 µg RNA on a 1.2% agarose gel.

RT-PCR analysis. The reverse transcriptase reaction was carried out according to the 'Protocol for First Strand cDNA Synthesis' of Fermentas using 5 µg total RNA, 0.2 µg random hexamer primers, 50 mM Tris/HCl (pH 8.3 at 25 °C), 50 mM KCl, 4 mM MgCl₂, 10 mM DTT, 20 U RiboLock RNase Inhibitor, dNTPs (10 mM each), RNase-free water and 200 U RevertAid M-MuLV reverse transcriptase (Fermentas). After the reverse transcriptase reaction, PCRs were carried out using the primers listed in Supplementary Table S2 in a 25 µl volume with 1 µl template (cDNA from the reverse transcriptase reaction described above), dNTPs (2.5 mM each), 50 pmol of each primer, 5% (v/v) DMSO, 10 mM Tris/HCl (pH 8.8 at 25 °C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 1.5 mM MgCl₂ and 1 U *Taq* DNA polymerase: denaturation at 94 °C for 2 min, 26 cycles with denaturation at 94 °C for 30 s, annealing at 64.5 °C (*novG* and *novS*), 60.7 °C (*novF*, *novH* to *novK*, *novO*), 58 °C (*novM*, *novP*, *novR*, *novU* to *novW*), 56.2 °C (*novL*, *novN*, *novT*) or 55 °C (*novE*, *novQ*) for 15 s, extension at 72 °C for 10 s, and a final elongation step at 72 °C for 7 min. The PCR product was checked by analysing 22 µl of the PCR with 8 µl loading buffer [50% (v/v) glycerol, 200 mM EDTA, 0.5% xylene cyanol (Sigma)] on a 1.2% agarose gel.

qRT-PCR analysis. qRT-PCR was carried out using the LightCycler RNA amplification kit SYBR Green I (Roche). Reaction mixtures were prepared using the primers listed in Supplementary Table S2 and following the manufacturer's instructions; however, the MgCl₂ concentration was reduced to 6.25 mM for investigations on *novE*, *novF*, *novG*, *novH*, *novO*, *novQ* and *gyrB^R*, and to 12.5 mM for investigations on *hrdB* and *novP*. The reverse transcriptase reaction was carried out for 20 min at 50 °C. Subsequently, the following temperature profile was utilized for amplification: denaturation at 95 °C for 30 s, followed by 45 cycles at 95 °C for 1 s (temperature transition, 20 °C s⁻¹), 60 to 55 °C (*novE*, *novG*, *novH* and *novO*), 58 to 53 °C (*hrdB* and *novQ*), 56 to 52 °C (*novF*, *novP* and *gyrB^R*) (step size, 0.7 °C; step delay, 1 cycle) for 10 s (temperature transition, 20 °C s⁻¹), and 72 °C for 13 s (temperature transition, 2 °C s⁻¹) with stepwise fluorescence acquisition at 60 to 55 °C, 58 to 53 °C and 56 to 52 °C in single mode. The number of copies of each sample transcript was then determined with the aid of LightCycler software and normalized to *hrdB*. The specificity of the PCR was verified by agarose gel electrophoresis on 2% agarose gels.

EMSAs. The methods used for the EMSAs are described in the supplementary material.

Nucleotide sequence accession numbers (for information). The nucleotide sequences of the genes and DNA regions used in this study are available in the GenBank database under accession numbers AF170880 (novobiocin cluster), AF205854 (novobiocin resistance gene *gyrB^R*) and NC_003888 (*hrdB*, locus tag SCO5820).

RESULTS

Insertion of transcriptional terminators and identification of promoter regions by RT-PCR analysis

A convenient method for the mapping of transcription units is the use of the Ω (omega) interposon, i.e. a DNA fragment containing an antibiotic resistance marker flanked by short inverted repeats which contain termination signals for transcription and translation (Prentki & Krisch, 1984; Raynal *et al.*, 2006). Using Red/ET-mediated recombination (Gust *et al.*, 2004), we inserted the 1.8 kb Ω aac cassette (Blondelet-Rouault *et al.*, 1997) into the coding sequence of the genes *novE*, *novF*, *novG*, *novH*, *novO* and *novS*. In each case, the cassette was inserted between nucleotides 3 and 7 of the coding sequence of the gene, i.e. replacing the second codon of the coding sequence. The insertions were carried out using cosmid nov-BG1 as target (Eustáquio *et al.*, 2005a). This cosmid contains the complete novobiocin cluster, as well as the integration functions of the phage Φ C31. The resulting cosmids with the inserted Ω aac cassettes were integrated into the genome of *S. coelicolor* M512 as described previously (Eustáquio *et al.*, 2005a). Southern blotting confirmed that in all integration mutants the entire cosmid had integrated site-specifically into the Φ C31 attachment site of the genomic DNA (data not shown).

When an *S. coelicolor* M512 strain carrying the intact novobiocin cluster was cultivated in production medium, RT-PCR experiments revealed the presence of transcripts for all genes of the novobiocin gene cluster (Fig. 2a). Controls without the reverse transcriptase confirmed that the detected signals were not due to contamination with genomic DNA.

Insertion of the Ω aac cassette into a transcription unit leads to termination of mRNA synthesis at the site of insertion. Transcription is reinitiated at the next active promoter sequence downstream of the Ω aac insertion site. As shown in Fig. 2(b, c and d), insertion of the Ω aac cassette into the second codon of *novE*, *novF* and *novG* led, as expected, to a complete abolition of the transcription of the affected genes themselves. In all three cases, however, transcripts of the adjacent gene, i.e. *novF*, *novG* and *novH*, respectively, were detectable, indicating the presence of promoter regions upstream of the latter genes. In contrast, insertion of Ω aac into *novH* led to a complete abolition of the transcription of *novHIJKLMN*, indicating that all of these

genes are part of a single transcription unit (Fig. 2e). Transcripts were detected, however, for *novO* and the genes downstream thereof, indicating the presence of a promoter region upstream of *novO*.

This finding prompted us to generate three additional mutant strains, carrying Ω aac insertions in *novO*, *novP* and *novQ*. In the first two cases, transcripts of the adjacent genes, i.e. *novP* and *novQ*, were detected (Fig. 2f, g), indicating that promoter sequences are located upstream of these genes. As shown by the subsequent qRT-PCR experiments (see below), only small amounts of transcripts are generated from the promoters upstream of *novO*, *novP* and *novQ*. Therefore, only weak bands are seen for most genes downstream of *novQ*, and transcripts of *novUVW* are below the detection limit. In the strain with the Ω aac insertion in *novQ*, transcription of the following genes was completely abolished, indicating that *novQ* forms a transcription unit with these genes (Fig. 2h). This was confirmed by the insertion of Ω aac into *novS*, resulting in a complete abolition of the transcription of *novSTUVW* (Fig. 2i).

Therefore, our experiments showed that promoter regions are located upstream of *novF*, *novG*, *novH*, *novO*, *novP* and *novQ*. Experimental evidence for the promoter activity of the DNA regions upstream of *novE* and *gyrB^R* has been published previously (Dangel *et al.*, 2008; Thiara & Cundliffe, 1989). Therefore, it can be concluded that at least eight DNA regions with promoter activity are present within the novobiocin cluster (Fig. 2k).

Fig. 2(h) appears to show a small amount of transcript for *novV*. However, we do not suggest that an additional promoter is present upstream of *novV*, as this band was only visible in this single experiment and not others, e.g. in Fig. 2(i).

The 16 genes from *novH* to *novW* are transcribed predominantly as a single polycistronic mRNA

Insertion of the Ω aac cassette into *novH* led to the complete termination of the transcription of *novHIJKLMN* (see above). Consistently, the strain carrying this insertion (hereafter called strain Ω novH) did not produce any detectable amounts of novobiocin (<0.2 mg l⁻¹), while a strain carrying the intact cluster produced 51 mg novobiocin l⁻¹. The genes *novQR*, situated further downstream of *novH*, direct the biosynthesis of the prenylated 4-hydroxybenzoate moiety, also referred to as 'ring A' of novobiocin (Pojer *et al.*, 2003a, b). If *novQ* and *novR* were well expressed in the Ω novH strain, we would expect an accumulation of the ring A moiety (see Fig. 1). However, the Ω novH strain accumulated only marginal amounts of ring A (<0.3 mg l⁻¹). This suggested that in the Ω novH strain, *novQ* and *novR* are only transcribed to a low extent. We confirmed this speculation by qRT-PCR experiments, comparing the numbers of transcripts of *novG*, *novH*, *novP* and *novQ* in *S. coelicolor* M512(nov-BG1), carrying the intact cluster, with those in *S. coelicolor* M512(nov-JH4),

carrying the Ω_{novH} cluster. In the latter strain the number of transcripts of *novH* was reduced to <1 % in comparison with the former strain, but also the transcripts of *novP* and *novQ* were reduced to 3 % in comparison with the former strain (Supplementary Fig. S1). This can be explained by the hypothesis that transcription of *novO*, *novP* and *novQ* (and of the genes located downstream thereof) is mainly controlled by the promoter located upstream of *novH*, which initiates a large transcript containing all 16 genes from *novH* to *novW*, while the promoter regions upstream of *novO*, *novP* and *novQ* have only minor relevance to the amounts of transcripts formed.

qRT-PCR investigations of the influence of *novE* and *novG* on the transcription of the novobiocin biosynthetic gene cluster

In order to investigate the influence of *novE* and *novG* on the transcription of the genes within the novobiocin cluster, we carried out qRT-PCR experiments. Suitable primer pairs were chosen for each of the eight genes located downstream of putative promoter sequences within the novobiocin cluster, i.e. *novE*, *novF*, *novG*, *novH*, *novO*, *novP*, *novQ* and *gyrB^R*. Reaction conditions, i.e. annealing temperature and $MgCl_2$ concentrations, were optimized for each primer pair until a linear relationship between the logarithm of the mRNA concentration and the cycle number was obtained over a concentration range of at least two orders of magnitude. The *hrdB* transcript, encoding the principal sigma factor of *S. coelicolor*, was used as an internal standard, and the number of transcripts for each sample was normalized to *hrdB*.

qRT-PCR analysis was then carried out, comparing the number of transcripts in three different strains. One strain carried the intact novobiocin cluster, and the two other strains carried clusters in which either *novE* or *novG* had been inactivated by an in-frame deletion (Dangel *et al.*, 2008; Eustáquio *et al.*, 2005b).

We first determined the time-course of novobiocin production (Fig. 3a). Only traces of novobiocin were detected within the first 48 h after inoculation, while the highest novobiocin production rate was observed between 72 and 96 h after inoculation. In contrast, dry cell weight increased between 24 and 72 h after inoculation (Siebenberg *et al.*, 2009), confirming the earlier observation that novobiocin production starts at the transition from growth phase to stationary phase (Kominek, 1972).

As may be expected, the highest amounts of transcripts for the novobiocin biosynthetic genes *novH*, *novO*, *novP* and *novQ* were detected immediately before the onset of novobiocin production, i.e. 48 h after inoculation (Fig. 3e–h). Thereafter, the amount of transcripts decreased, but they were still clearly detectable after 72 and 96 h, i.e. in the early stationary phase. Expression of all four genes was perfectly synchronous, which is in accordance with the hypothesis that all these genes are transcribed as a single

operon. During the active growth phase, i.e. 32 h after inoculation, hardly any transcripts were found for these four genes.

In contrast, transcripts for the two putative regulators *novE* and *novG* could clearly be detected at 32 h after inoculation, indicating that the expression of the regulatory genes (especially *novE*) is initiated earlier than the expression of the biosynthetic enzymes (Fig. 3b, d).

For the resistance gene *gyrB^R*, a first maximum of transcripts was detected after 48 h, i.e. synchronous with the expression of *novH* and *novOPQ*. It is tempting to speculate that this may be caused by co-transcription of *gyrB^R* with the genes from *novH* to *novW* on a single mRNA. In sharp contrast to *novH* and *novOPQ*, however, the amount of transcripts for *gyrB^R* increased again after 72 h, i.e. at the time when novobiocin concentration started to rise. This is in accordance with the results of Thiara & Cundliffe (1989), who cloned the promoter region of *gyrB^R* into a promoter probe vector and showed (by expression in *Streptomyces lividans* TK24) that the promoter was induced by cultivation in the presence of novobiocin. Thiara & Cundliffe suggested that this induction is mediated by the change of superhelical density of chromosomal DNA (i.e. loss of negative supercoils), caused by the gyrase inhibitor novobiocin.

In contrast to the strain with the intact cluster, the two strains with in-frame deletions in the putative regulators *novE* and *novG* showed only marginal novobiocin production (Fig. 3a). Seven days after inoculation, the strain with the intact cluster had accumulated 45 mg novobiocin l^{-1} in this experiment, whereas the $\Delta novE$ and $\Delta novG$ strains had produced less than 0.4 mg l^{-1} . The amounts of transcripts for the novobiocin biosynthetic genes *novH* and *novOPQ* were dramatically reduced in the $\Delta novE$ and $\Delta novG$ strains in comparison with the strain with the intact cluster (Fig. 3e–h), proving that *novE* and *novG* act as transcriptional regulators in novobiocin biosynthesis. Notably, *novG* transcription was also strongly reduced in the $\Delta novE$ strain: at 48 h after inoculation, the amount of transcript was only 5 % of that observed in the strain with the intact cluster (Fig. 3d). This suggests that *novG* expression is largely, though not entirely, dependent on the presence of *novE*. In contrast, *novE* expression was still quite high in the $\Delta novG$ strain (Fig. 3b), suggesting that *novE* expression is not dependent on *novG*.

The predicted gene product of *novF* shows high sequence similarity to prephenate dehydrogenases and is therefore expected to supply 4HPP, the common precursor of both the prenylated 4-hydroxybenzoate moiety and the amino-coumarin moiety of novobiocin (Fig. 1). Transcription of *novF* is remarkably similar to that of *novE* in the strain with the intact cluster and in the $\Delta novG$ strain (Fig. 3c), which indicates either close co-regulation or even co-transcription of both genes.

Fig. 3. qRT-PCR analysis of *S. coelicolor* M512(nov-BG1) containing the entire novobiocin biosynthetic gene cluster, *S. coelicolor* M512(nov-VD2) containing the *novE*-defective cluster and *S. coelicolor* M512(nov-AE10) containing the *novG*-defective cluster. (a) Production of novobiocin. (b–i) qRT-PCR analysis of the expression of selected genes of the novobiocin biosynthetic gene cluster.

Construction of an optimized *novG* expression vector for novobiocin overproduction

The results described above suggest that control of transcription from the promoter upstream of *novH* is the central mechanism for regulation of novobiocin biosynthesis. We tried to utilize this knowledge in order to increase novobiocin yields.

Transcription from the promoter upstream of *novH* is positively regulated by the DNA-binding protein NovG. The NovG binding site has been identified in the intergenic region between *novG* and *novH*. The palindromic sequence of this binding site (Fig. 4a) begins 2 bp downstream of the *novG* stop codon, i.e. 194 bp upstream of the *novH* start codon (Fig. 4a).

Using a previously constructed *novG* expression vector, pAE8 (Eustáquio *et al.*, 2005b), we could increase novobiocin production in the heterologous novobiocin production strain 2.9-fold in comparison with the empty vector control. However, pAE8 contained not only the coding sequence of *novG* but also 135 bp of the intergenic region downstream of *novG*, i.e. it included the NovG binding site (Fig. 4b). We speculated that this was not an optimal construct to increase novobiocin production, since much of the translated NovG protein would bind to the NovG binding site in the multicopy plasmid pAE8, leaving only a fraction of the produced NovG available for binding within the novobiocin cluster. Therefore, we constructed a new *novG* expression plasmid, named pAE12, which contained just 1 bp of the intergenic region downstream of *novG*, and therefore did not contain the NovG binding site (Fig. 4c). Otherwise, this plasmid was identical to pAE8. Transformation of this plasmid into a heterologous expression strain carrying the intact novobiocin cluster resulted in 8.4-fold overproduction of novobiocin compared with the empty vector control. This is the most effective genetic method for the stimulation of novobiocin production identified so far.

Fig. 4. (a) Schematic representation of the regulator gene *novG* and the binding site of its gene product. (b, c) Inserts of the *novG* overexpression plasmids pAE8 and pAE12.

DISCUSSION

The present study provides evidence for the role of *novE* and *novG* in the transcriptional regulation of novobiocin biosynthesis.

qRT-PCR experiments showed that effective transcription from the promoter upstream of *novH* depends on the presence of *novG* (Fig. 3). Transcription from this promoter may result in a large polycistronic mRNA containing all 16 genes from *novH* to *novW* which together direct all steps of novobiocin biosynthesis from glucose 1-phosphate and 4-HPP or tyrosine (Fig. 1). Possibly, even the resistance gene *gyrB^R* is included in this transcript, extending it to a size of 20 kb. Transcripts of this size are not unusual in secondary metabolic gene clusters, as the coding sequences of modular polyketide synthase or non-ribosomal peptide synthase genes often span even larger DNA regions (Fischbach & Walsh, 2006).

Within the suggested large operon starting with *novH*, RT-PCR had indicated the presence of internal promoters upstream of *novO*, *novP* and *novQ*. Internal promoters within operons have been identified previously in *Streptomyces*, e.g. *galP2*, a low-level constitutive promoter internal to the galactose operon in *S. lividans* and *S. coelicolor* A3(2) (Westpheling & Brawner, 1989). A recent study (Laing *et al.*, 2006) suggests that in *S. coelicolor* (in contrast to *E. coli* and *Bacillus subtilis*), expression levels of the individual genes of an operon decrease with increasing distance from the transcription start, and suggests that the frequently encountered internal promoters ensure adequate transcription of the terminal genes of an operon. Under our experimental conditions, however, transcription from the internal promoters upstream of *novO*, *novP* and *novQ* was low in the Ω *novH* strain, and these promoters may not have a significant role in novobiocin biosynthesis.

For the first time, to our knowledge, our study shows that effective transcription of *novG* depends on the presence of *novE* (Fig. 3d). This suggests a cascade-like regulation mechanism of *novE* and *novG*, i.e. *novE* triggers transcription of *novG*, which in turn triggers transcription of the novobiocin biosynthetic genes. Consistent with this hypothesis, novobiocin formation in a *novE*-defective mutant could be restored by an intact copy of *novG*, while novobiocin formation in a *novG*-defective mutant remained low even after expression of *novE* (Dangel *et al.*, 2008).

While we could prove that *novE* positively regulates transcription of *novG*, the precise mechanism of this regulation is as yet unknown. We could not demonstrate binding of the NovE protein to the DNA region upstream of *novG* (Dangel *et al.*, 2008). Further proteins may be involved in the regulation by NovE. Also, the mechanism which controls transcription of *novE* is unknown. It should be noted, however, that *novE* contains a TTA codon, which is rare in *Streptomyces*. Expression of *novE* therefore depends on the expression of the gene for the UUA-specific

tRNA *bldA*, which appears to be involved in the regulation of secondary metabolism in streptomycetes (Chandra & Chater, 2008).

Notably, our RT-PCR results did not allow us to distinguish between low and high expression of the biosynthetic genes. Only the subsequent qRT-PCR revealed a correct picture of the amount of transcripts of the genes of novobiocin biosynthesis.

All experiments in the present study were carried out in a heterologous producer strain, which carries the novobiocin gene cluster integrated into the $\Phi C31$ attachment site of the chromosome. As described for the genuine producer strain (Kominek, 1972), novobiocin production in the heterologous producer strain started at the transition from the growth phase to the stationary phase, suggesting that *novE* and *novG* expression was regulated in a similar timely fashion as in the genuine novobiocin producer strain. The mechanism that triggers expression of *novE* and *novG* remains to be elucidated for both strains.

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